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The research supported by this award is based on the hypothesis that the interfilament distances between microtubules and neurofilaments are maintained by a polymer brush based mechanism, that results in what has been called colloidal stabilization. We suggest that failure of such stabilization may be related to, and even causal, in neuropathologies such as amyotrophic lateral sclerosis (ALS) and Alzheimer's disease. Thus focus of the research in this proposal is a series of experiments that test the polymer brush based mechanism. These experiments include atomic force microscopy, macromolecular exclusion measurements on isolated neurofilaments and microtubules and analysis of neurofilament distributions in electron micrographs. With an understanding of the basic mechanism, conditions under which the colloidal stabilization fail will be examined. The main findings to date are that macromolecular exclusion measurements and analysis of neurofilament distributions in electron micrographs are consistent with a polymer brush based long range repulsive interaction, thus supporting the main hypothesis. The results from this research will provide fundamental insight into the mechanism of neurodegeneration, and suggest new approaches to therapies and treatments for neuropathologies.

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INTRODUCTION

The research supported by this award is based on the hypothesis that the interfilament distances between microtubules and neurofilaments are maintained by a polymer brush based mechanism, that results in what has been called colloidal stabilization. We suggest that failure of such stabilization may be related to, and even causal, in neuropathologies such as amyotrophic lateral sclerosis (ALS) and Alzheimer's disease. Thus focus of the research in this proposal is a series of experiments that test the polymer brush based mechanism. These experiments include atomic force microscopy, macromolecular exclusion measurements on isolated neurofilaments and microtubules and analysis of neurofilament distributions in electron micrographs. With an understanding of the basic mechanism, conditions under which the colloidal stabilization fail will be examined. The results from this research will provide fundamental insight into the mechanism of neurodegeneration, and suggest new approaches to therapies and treatments for neuropathologies.

BODY OF REPORT

Below the approved statement of work is divided into four sections, and research progress associated with the tasks in each section is reported.

1. From Statement of work (verbatim): "The contractor will isolate microtubules, neurofilaments and associated proteins from bovine tissue. These proteins will be characterized by SDS-polyacrylamide gel electrophoresis, imaging by atomic force microscopy (AFM) and appropriate physical/biochemical methods. Isolation of these proteins will occur regularly throughout the three years of the proposed contract, as required for the specific experiments proposed. In the first year methods for immobilizing microtubules with microtubule associated proteins (MAPs) onto solid supports will be established. AFM force measurements will be performed on microtubules with MAPs to establish if the MAPs behave as an entropic brush. The data will be analyzed using isoforce difference mapping and fitting to appropriate theoretical models. Controls for these force measurements will include microtubules assembled in the absence of MAPs and microtubules for which MAPs have been chemically removed."

We have established methods for isolating neurofilaments from bovine spinal cord and microtubules from bovine brain (material obtained from a local slaughter house). Microtubules were prepared as described by Williams and Lee (1982). Fresh bovine brains obtained at a local slaughter house are cubed and put on ice to cool (and for transport back to the lab). An equal weight of a suspension buffer (50 mM imidazole, 0.5 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM EGTA, pH 7.2-7.25) is added, and the brains are homogenized in a precooled Teflon Dounce type glass homogenizer for 2 passes at 3000 rpm. The homogenate is centrifuged in GSA rotor at 12000 rpm for 60 minutes at 2° C, and the supernatant is collected. PMSF at 1 mM is used to reduce proteolysis. To the volume of supernatant, 0.025x volume of a buffer (pH 6.8-6.9)

containing 1 M imidazole, 10 mM $MgCl_2$, 2 mM EDTA, and 20 mM EGTA, 0.5x volume glycerol and GTP to 1 mM are added. The solution is warmed to 37°C and incubated for 30 minutes. Polymerized microtubules are sedimented by centrifugation in Ti45 rotor at 40000 rpm for 60 minutes at 25°C. The pellet is resuspended in the suspension buffer at pH 6.7-6.8, and made 1 mM EGTA and 1 mM GTP. The mixture is cooled for 20 minutes at 5°C to depolymerize the microtubules. This solution is then centrifuged in Ti60 rotor at 42000 rpm for 35 minutes at 5°C, and the supernatant collected. This warm-cold cycle is repeated two more times, resulting in a yield of >100 mg of microtubules/MAP2 per brain used. The preparation is aliquoted, rapidly frozen and stored at –80 °C. Neurofilaments are isolated from bovine spinal cord also obtained at a local slaughterhouse (Troncoso et al., 1990). Diced bovine spinal cord is homogenized with buffer A (100 mM MES, 1.0 mM EGTA, and 0.5 mM MgCl2 at pH 6.5) in a concentration of 10 g of cord/16 ml of buffer. The homogenate is centrifuged at 34500*g* for 30 min at 4 °C. The supernatant will be mixed to 30% glycerol and incubated at 37 °C for 20 min. The incubation mix is then centrifuged at 158000*g* for 120 min at 20 °C. The pellet is resuspended in buffer C (100 mM MES, 1.0 mM DTT, and 170 mM NaCl at pH 6.5) for imaging as native neurofilaments. For preparation of homopolymers of NF-L, pellets of native filaments are disassembled in buffer B (6 M urea, 1.0 mM DTT, 25 mM NaH_2PO_4, and 1.0 mM EGTA at pH 7.5); the subunits were separated by HPLC (Pharmacia Mono-Q HR 5/5) with a linear gradient of 0.5 M NaCl. SDS-PAGE will be used to check the content and purity of the eluate. NF-L homopolymers are reassembled by open dialysis of the purified NF-L subunits against buffer C.

These preparations have the expected (known) physical properties (i.e. they are large volume gelatinous pellets) and SDS-polyacrylamide gel electrophoresis confirms that the correct proteins are present. Using these preparations we have examined a range of immobilization methods for microtubules. For pure microtubules two methods, silane modification or polylysine modification of a solid support allows for robust immobilization and AFM imaging. For the silanization, a mica substrate is submerged in a solution of 1 mM acetic acid containing 1% (v/v) neat aminopropyltriethoxysilane for

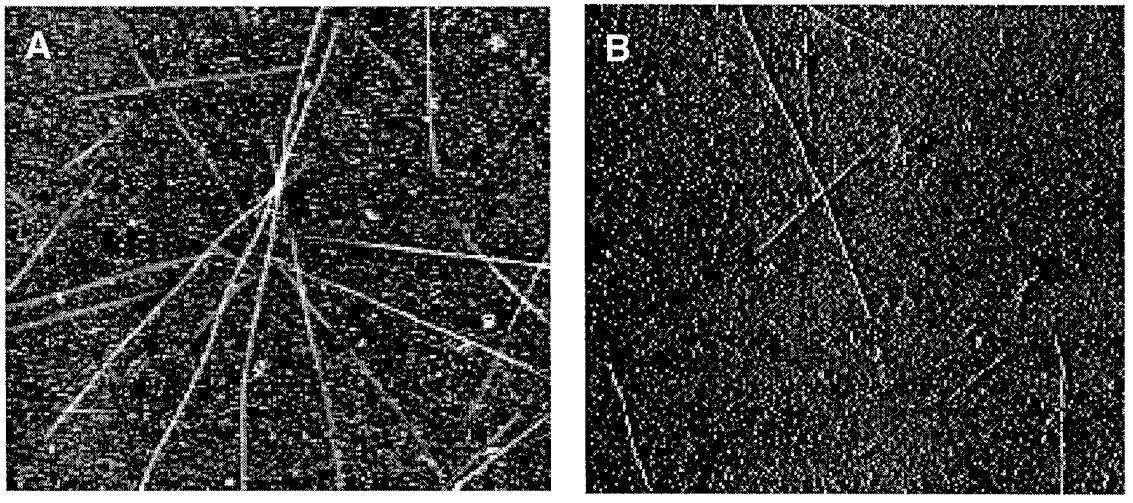

Figure 1. AFM images of microtubules on A) silane modified mica and B) polylysine modified mica. A) is a height image and B is a deflection (error signal) image.

10 minutes. The substrates are then rinsed thoroughly with water and air dried. A suspension of approximately 1 ug/ul of polymerized microtubules are incubated on the substrate, resulting in binding that allows AFM imaging (Figure 1). For the polylysine coating, 100 ul of 0.12 mg/ml polylysine (90 kDa) is pipetted onto a mica substrate. The polylysine is allowed to adsorb for >20 minutes, and excess polylysine is removed by extensive rinsing with 20 mM NaCl. Again, a suspension of approximately 1 ug/ul of polymerized microtubules are incubated on the substrate, resulting in binding that allows AFM imaging (Figure 1). While these methods work well with pure microtubules, in the presence of microtubule associated proteins the microtubules do not sufficiently well attached to allow for force volume imaging. When force curves are collected on these samples they dislodge from the surface. Thus we continue to work to improve the immobilization methods to allow for force volume data collection.

2. From statement of work (verbatim): "In the second year macromolecular exclusion experiments will be performed on microtubules with MAPs. The ability of the microtubules/MAPs to exclude macromolecules will be determined by a centrifugation-based approach. Fluorescent macromolecular probes will be used to determine the effective volume of the microtubules/MAPs, in order to establish if MAPs act as an entropic brush. The size dependency of the exclusion properties of the microtubules with MAPs will be determined by using a series of varying probe molecule size. Controls for these force measurements will include microtubules assembled in the absence of MAPs and microtubules for which MAPs have been chemically removed. If appropriate, similar experiments will be performed with neurofilaments."

We are ahead of schedule on this part of the project (initially scheduled to begin in year two), and have made some progress on several fronts with regard to macromolecular exclusion. To begin with, preliminary macromolecular exclusion experiments using fluorescently labled dextrans show that large dextrans are excluded from the proximity of microtubules with MAPs. These results are consistent with and support the proposed colloidal stabilization model.

The problem macromolecular exclusion in neurofilaments is also being addressed by an alternative approach, that complements the molecular exclusion measurements. This alternative approach is based on analysis of electron micrographs of axonal cross sections (Figure 2). Such micrographs have been provided to us by Dr. Bruce Trapp of the Cleveland Clinic. When motor neurons are sectioned and visualized by electron microscopy, the distribution of neurofilaments in the cross section contains information about the interactions this research seeks to understand. If the neurofilaments interact strongly, one expects a highly ordered distribution, whereas if they interact very weakly, one expects a random distribution.

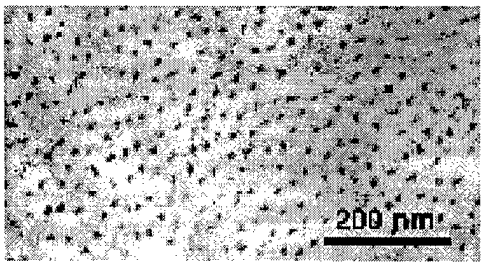

Figure 2. Electron micrograph of neurofilaments in mouse motor neuron used to determine potential of mean force between filaments.

While several attempts have been made to quantify the degree of order in these distributions (Price et al., 1988; Price et al., 1993; Hsieh et al., 1994), there have been no reported attempts to explicitly relate this order to interfilament potentials. We have applied an information theory-based approach to extract interfilament potentials of mean force from these electron micrographs. This method has been shown to accurately predict solute hydration energies in water (Garde et al., 1996) and should be directly applicable to neurofilament distributions. In this method, the neurofilament positions are digitized and sent to a computer program which radomly lays many observation circles of a defined size over the micrograph. Each time a circle is placed, the program counts the number of neurofilaments in the circle. A distribution of probabilities of finding a given number of neurofilaments within an observation circle is then calculated. For mouse sciatic nerves we find that this distribution is Gaussian, which suggests that the two-body interactions between the neurofilaments are the dominant type of interaction. Further, the mean of this distribution is proportional to neurofilament density and the standard deviation is inversely related to neurofilament order and hence the strength of the interfilament potential. Using this approach we have also examined micrographs from mice in which the myelin-associated glycoprotein (MAG) has been disrupted (Yin et al., 1998). Relative to controls, these mice develop a chronic motor neuropathy that is characterized by reductions in neurofilament spacing as well as reduced axonal caliber and neurofilament phosphorylation. Compared to controls, MAG knockouts have greater NF densities yet less spatial order (i.e., weaker interactions), as evidenced by the broader, right-shifted distribution curve (Fig). This surprising result suggests that NF expression is inversely related to the strength of interfilament interactions.

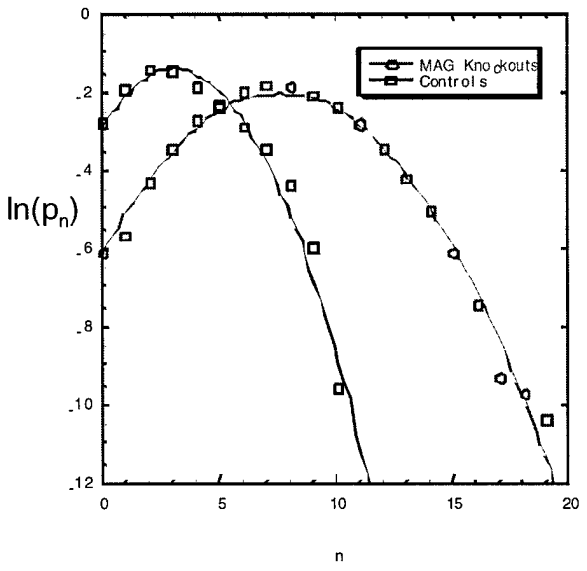

Figure 3. Distribution of probability of occupancy for control and MAG knockout mice at 9 months, observation circle 60 nm radius. n is the number of NF's per circle and p_n is the probability of finding n NF's in a given circle. Lines are quadratic curve fits (equivalent to Gaussian for these semilog axes).

3. From statement of work (verbatim): "In year three the conditions and treatments that modulate the effect of the entropic brush of microtubules and neurofilaments will be established. The effect of varying concentrations of monovalent salt, divalent salt and pH on the entropic brush, as assayed by AFM force measurements and macromolecular exclusion experiments, will be determined. Using the results from these experiments appropriate combinations of ionic conditions, or new conditions, will be examined. The effect of phosphorylation state of neurofilaments on the entropic brush will be determined. Neurofilaments will be enzymatically dephosphorylated or hyperphosphorylated and the effect of the entropic brush will be examined as

above. Neurofilaments will be treated with aluminum to determine if this metal directly effects the entropic brush."

In year one reported here there was no research on work scheduled for year three of the award.

4. From Statement of work (verbatim): "The results from the work will be reported to the appropriate office at the USAMRMC at times specified by the USAMRMC. The results from the work will also be published in international peer reviewed scientific journals and presented at national meetings. The presentations will be on an annual basis, and the publication will be as appropriate with regard to content of the work."

The present document represents the required annual report to the USAMRMC. In addition, the research supported by this award has been presented at several meetings and seminars as listed below under reportable outcomes. To date there are no publications

KEY RESEARCH ACCOMPLISHMENTS

- Isolation of neurofilaments from bovine spinal cord.

- Isolation of microtubules from bovine brain.

- Successful immobilization of microtubules for AFM imaging.

- Finding that microtubules with native microtubule associated proteins (MAPs) exclude fluorescently labled dextrans consistent with a polymer brush based colloidal stabilization model for microtubules.

- Finding from analysis of electron micrographs of axonal cross sections that the distribution of neurofilaments is consistent with a long range repulsive force in which the two body interaction term dominates. This supports the proposed model.

- Finding that in the neuropathological condition associated with disruption of the myelin associated glycoprotein, neurofilament spacing decreases and the degree of order decreases.

REPORTABLE OUTCOMES

Presentations/seminars by Dr. Hoh that include work supported by this award
National Institutes of Health
National Institute of Neurological Orders and Stroke
Analytical Cell Biology Department

North Carolina State University
Department of Material Science

The Johns Hopkins Protein Folding Meeting
Coolfont Resort, WV

Nanoscience and Nanotechnology Symposium
University of Pennsylvania

Tristate Biomedical Engineering Meeting
Drexel University

EMSL2000
Pacific Northwest National Laboratory
Environmental Molecular Sciences Laboratory

Funding applied for based on work supported
Title: Protein-based polymer brushes: modeling and simulation of nanostructures
Source: National Science Foundation
Amount (total costs): $2,100,000
Duration: 3 years
Co-investigators: Dr. Michael Pauliatis; Dr. Thomas Woolf; Dr. William Russell
Submitted: 4/10/2000
The aim of this proposal is to develop a predictive theoretical framework for the protein based polymer brush model which is being studied experimentally in microtubules and neurofilaments in the supported work.

Employment opportunities received on experiences supported by this award
Dr. Jan Hoh was promoted to Associate Professor in part do to his activities associated with this award.

CONCLUSIONS

Specific findings are noted above. The main overall conclusion from the first year of work under this award are that the data supports the polymer brush based colloidal stabilization model. This justifies continued research in order to examine

Work on this project also suggests that the project would benefit from expanding in three directions. First, it would be useful to bring additional experimental methods and approaches to bear on the problem of interfilament spacing. To this end we plan to explore neutron scattering as a complementary approach to the AFM force measurements and the macromolecular exclusion experiments for examining interfilament forces/exclusion. Such measurements can provide direct interfilament spacing measurements in neurofilament/microtubule gels and can also provide

potentials of mean force in dilute solutions. We have secured beam time at the National Institute of Standards and Technology Neutron Facility in Gaithersburg in July 2000 for preliminary experiments of this type. Second, the recent development of single molecule force spectroscopy has opened the door to direct examination of polymer properties of microtubule associated proteins. It is these properties that underlie the proposed colloidal stabilization model. Thus experiments to directly determine these properties would be important. Third, it has become clear that the theory for understanding polymer brush type behavior in proteins as proposed for neurofilaments and microtubules is entirely inadequate. With the resources provided under this award we can not undertake a major effort in this direction. Thus, while we are attempting to deal with as much of the theory as possible without detracting from the experimental work that is at the center of the statement of work, a larger effort in this area would require a separate grant. For this purpose have applied for a grant from the National Science Foundation.

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